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Background: Fibroblast-like synoviocytes (FLS) are pivotal in inflammation and joint damage of rheumatoid arthritis (RA). These cells acquire an aggressive and invasive phenotype and secrete inflammatory mediators, metalloproteases and cathepsins that perpetuate inflammation and lead to cartilage and bone damage. We have previously shown that non-canonical Wnt5a pathway is involved in the aggressive phenotype of FLS by increasing their migration and invasion ability, and by stimulating the inflammatory response. The non-canonical Wnt signaling pathway included the planar cell polarity (PCP), with the activation of Rho and Rac GTPases, and the Wnt/Ca2+ pathways. We have also shown that Wnt5a contributes to the aggressive phenotype of RA FLS by binding to RYK receptor, through Rho-ROCK pathway and the activation of MAPKs, ERK and P38, as well as the activation of AKT and GSK3 β .

Objectives: To elucidate the therapeutic potential of the ROCK inhibitor (Y-27632) in the K/BxN serum transfer arthritis model.

Methods: Two groups of C57BL/6J mice were used, in the control group, mice were treated with physiological serum and in the experimental group with a ROCK inhibitor (Y-27632). Arthritis was induced by intraperitoneal injection of 100 μ l of K/BxN serum on days 0 and 2. In the experimental group, mice were treated with intraperitoneal injections of 10 mg/kg from day 0 until sacrifice, on day 10. Control mice were treated with the same volume of physiological serum. Arthritis was assessed by two observers using a semiquantitative clinical score. For histological analysis, it was decided to obtain the right ankle joints and foot. Joints were fixed in formalin for 6h and were decalcified and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and toluidine. Finally, total RNA was obtained from wrist and ankle joints of mice and the expression of inflammatory mediators and metalloproteases was analyzed by real-time PCR.

Results: Arthritis was induced in C57BL/6J mice, which were treated with Y-27632 (ROCK inhibitor) or with physiological serum. The incidence of arthritis was 100% in both groups of mice and there were no differences in the course of the disease. Clinical score was significantly lower in the Y-27632-treated mice, all along the follow-up, compared with controls. Similar results were observed in the histological analysis. We also analyzed the effect of ROCK inhibitor on the inflammatory response of K/BxN serum-transfer induced arthritis. This analysis revealed that expression of IL6, IL1 β , CXCL1, MMP3, MMP9 and MMP13 were significantly decreased in Y-27632-treated mice compared with control mice. In addition, TNF and NOS2 expression was reduced in Y-27632-treated mice to reach the same levels that observed in C57BL/6J mice without arthritis.

Conclusion: These results indicate that the inhibition of the Rho-ROCK pathway decreases the severity of arthritis in the K/BxN serum transfer model, and point to ROCK as potential therapeutic target for RA. Supported: ISCIII / PI17 / 01660 / RETICS Program, RD16 / 0012/0014 / Cofinanced FEDER.

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SAT0016

DEVELOPMENT OF FIBROBLAST-LIKE SYNOVIOCYTE ASSAYS FOR TARGET DISCOVERY IN RHEUMATOID ARTHRITIS

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Background: The rheumatoid arthritis (RA) synovium is characterized by an overabundance of fibroblast-like synoviocytes (FLS), which play a central role in the initiation and perpetuation of disease via multiple mechanisms.¹ FLS promote disease progression by producing high levels of proinflammatory factors, migrating to and invading cartilage and bone, and promoting self-proliferation and resistance to apoptosis. Our current understanding of the molecular mechanisms that govern FLS-mediated pathology in the synovial joint remains incomplete. Importantly, almost 30% of treatment-naïve early RA patients exhibit a strong fibroid phenotype that correlates with relatively poor response to disease-modifying anti-rheumatic drugs.² Yet, current therapies in RA are not directly aimed at FLS pathology, creating an opportunity for novel therapeutic target discovery.

Objectives: Our aim is to develop a broad suite of screening-amenable assays in RA patient-derived FLS for the discovery of target pathways that control multiple pathological properties, including cytokine secretion, migration, and invasion.

Methods: A sensitive high-throughput RA-FLS secretion assay was developed to examine the ability of small-molecule inhibitors to block the production of interleukin (IL)-6 and matrix metalloproteinase (MMP)-3 in response to stimuli. To create a physiologically relevant stimulus, a surrogate synovial fluid cocktail (composed of 12 factors) was defined and titrated for optimal concentration selection. Small-molecule inhibitors (N=170) of diverse biological pathways were screened using the full cocktail or individual stimulation (TNF α , IL-1 α , or IL-17) to characterize assay performance. In addition, an FLS platelet-derived growth

factor (PDGF)-mediated migration screening assay was developed using a live cell imaging system (Incucyte) to quantify real-time FLS migration.

Results: Due to the variability and limited volume of synovial fluid, we developed a surrogate synovial fluid cocktail to mimic the relevant stimulation of RA-FLS in the inflamed joint. The surrogate cocktail was composed of 12 factors: TNF α , IL-1 α , IL-17, IFN γ , OSM, LIF, GM-CSF, IP-10, VEGF, PDGF, AREG, and FGF2. Individual titration of these factors demonstrated that only 3 stimulatory factors (TNF α , IL-1 α , and IL-17) resulted in a robust increase of IL-6 production. Importantly, when all 12 factors were combined, a synergistic increase in IL-6 and MMP-3 production by FLS was observed. Screening results identified several reference compounds, including an inhibitor of transforming growth factor- β -activated kinase 1 (TAK1), that was previously reported to block cytokine secretion in FLS.³ Treatment with this compound showed complete inhibition of IL-6 and MMP-3 secretion. In addition to the cytokine secretion assay, treatment of FLS with this TAK1 inhibitor resulted in almost complete inhibition of migration (Fig. 1).

Conclusion: Novel FLS assays were developed to discover new targets and interrogate pathways involved in multiple disease-driving mechanisms of FLS in RA. In order to mimic the inflammatory environment present in the RA synovium, we developed a 12-factor surrogate synovial fluid cocktail. A synergistic release of both IL-6 and MMP-3 was demonstrated following cocktail stimulation compared to individual cytokines. This points to the important contribution that multiple factors play in the FLS pathogenic processes and will allow us to uncover pathway interactions that may not be captured with single stimuli. In addition, the development of a real-time, 96-well, imaging-based assay to interrogate FLS migration will allow us to identify targets that control this critical pathological function of FLS.

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Figure 1. Treatment of RA-FLS with TAK1 inhibitor over 24h demonstrates complete inhibition of PDGF-mediated migration.

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SAT0017

METABOLIC CHANGES INDUCED BY ANTI-MALONDIALDEHYDE/MALINDIALDEHYDE-ACETALDEHYDE ANTIBODIES PROMOTE OSTEOCLAST DEVELOPMENT

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Background: Malondialdehyde (MDA) is a highly reactive compound produced by lipid-peroxidation in situations associated with oxidative stress. MDA can irreversibly modify proteins residues such as lysine, arginine and histidine. In addition, MDA adducts can further react with acetaldehyde to generate malondialdehyde-acetaldehyde (MAA) modifications. Such modifications can give rise to immunogenic neo-epitopes that are recognized by autoantibodies. In fact, anti-MDA/MAA IgG antibodies are significantly increased in the serum of patients with autoimmune diseases, such as rheumatoid arthritis (RA) (1) and systemic lupus erythematosus (2). Recently, we have shown that anti-MDA/MAA IgG antibodies are able to promote osteoclast (OC) differentiation *in vitro* (1).

Objectives: To investigate the molecular mechanisms triggered by anti-MDA/MAA autoantibodies during osteoclastogenesis.

Methods: OCs were generated from monocyte-derived macrophages in the presence of the cytokines RANK-L and M-CSF. The development of OCs was monitored by light microscopy following tartrate-resistant acid phosphatase (TRAP) staining and erosion area on synthetic calcium phosphate-coated plates. Three different recombinant human monoclonal anti-MDA/MAA antibodies, cloned from single synovial B cells of RA patients, control antibodies and Fab fragments of the antibodies were added to OC cultures. Glycolysis was inhibited by 2-deoxyglucose, and Fc-gamma receptor I or II by anti-CD64 or anti-CD16 neutralizing antibodies. IL-8 levels were measured by enzyme linked immunosorbent assay. Cellular metabolism was monitored using Seahorse XF Analyzer (extracellular acidification rate and oxygen consumption) and a colorimetric L-Lactate assay.

Results: Lactic acid production correlated with the osteoclastogenic effect of some but not all anti-MDA/MAA antibodies on OCs ($R=0.4758$, $p=0.0252$) suggesting an antibody-mediated regulation of glycolysis. Further, extracellular acidification (ECAR) and oxygen consumption (OCR) rate of the developing OCs were increased by the osteoclastogenic anti-MDA/MAA clones (maximum increase of 54% for the ECAR and 78% for the OCR by clone 146+01G07, and maximum increase of 28% for the ECAR and 39% for the OCR by clone 1103:01H05), but not by the non-osteoclastogenic anti-MDA/MAA clones or control antibodies. The glycolysis inhibitor 2-deoxyglucose completely abolished the osteoclastogenic effect of the anti-MDA/MAA clones at drug concentrations that did not influenced baseline OC development. Fab2 fragments of the osteoclastogenic anti-MDA/MAA clones had no effect on OC development and metabolism. In accordance with this, Fc-gamma receptor I neutralizing antibodies completely abolished the osteoclastogenic effect of the anti-MDA/MAA clones. The osteoclastogenic effect of the anti-MDA/MAA antibodies was independent of IL-8 production. In contrast to anti MDA/MAA antibodies, ACPA-mediated osteoclastogenesis was independent of glycolysis and Fc-gamma receptors but dependent on IL-8.

Conclusion: Our results describe a novel glycolysis-dependent mechanism by which anti-MDA/MAA antibodies promote osteoclast development that is different from the one previously described for ACPA.

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SAT0018

THE THERAPEUTIC EFFECT OF STIMULATION OF THE SPLENIC NEUROVASCULAR BUNDLE IN MICE WITH COLLAGEN-INDUCED ARTHRITIS IS ENHANCED BY CONCOMITANT ANTI-TNF TREATMENT

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Background: Vagus nerve (VN) stimulation has shown the potential to improve the disease development in animal models of arthritis and in patients with RA. However, the VN can affect respiratory, cardiovascular, endocrine and gastro-intestinal physiology. The splenic nerve (SpN) has been confirmed to be the principal effector nerve for the VN-mediated immune control. Previous studies have shown that stimulating the splenic nerve resulted in an increase of norepinephrine in the spleen, as well as a significant reduction in LPS-induced TNF (1).

Objectives: To test the therapeutic efficiency of splenic nerve stimulation (SNS) in collagen-induced arthritis (CIA) in mice alone or in combination with anti-TNF treatment.

Methods: CIA was induced in DBA1/J mice by immunization with bovine type II collagen at days 0 and 21. At day 11, mice were implanted with micro-cuff electrode (CorTec) onto the SpN or VN. From day 16 to day 45, SNS were applied as rectangular charged-balanced biphasic pulses with 650 μ A pulse amplitude, 200 μ s pulse width at 10 Hz frequency for 2 min 1 or 6 times a day using a Plexon stimulator. In order to investigate the mechanism of action in more detail, propranolol, a beta-adrenergic receptor (β -AR) antagonist, was added to the drinking water of mice receiving SNS. In addition, a control group was treated with anti-TNF (etanercept, 3 times/week; 10mg/kg i.p.). In curative settings, SNS and/or anti-TNF treatment was applied starting when mice scored positive for 3 consecutive days. Clinical arthritis was determined by visual examination of swelling and redness of the paws and measurement of paw thickness. Sham mice were undergoing the same procedure but did not receive stimulation.

Results: In CIA in mice all sham animals developed arthritis, compared to only 14% following six times per day SNS ($p < 0.001$) in a prophylactic setting. In contrast, 85% of the animals developed arthritis ($p = 0.35$) when SNS was applied only once a day. In both stimulated groups a significant decrease in clinical scores and paw thickness was observed compared to unstimulated group ($p < 0.01$ and $p < 0.05$, respectively). While etanercept treatment reduced clinical scores ($p < 0.001$) an immediate rebound in clinical score was seen following treatment arrest, while mice with SNS were still partially protected 35 days after treatment discontinuation ($p = 0.013$, compared to sham). Propranolol inverted the effect of SNS in CIA mice. Finally, when SNS was applied as a curative treatment, clinical scores were significantly reduced ($p < 0.001$). Importantly, these clinical scores even further decreased when anti-TNF treatment was given to mice receiving SNS.

Conclusion: These studies demonstrate that SNS suppresses pro-inflammatory cytokine production, and reduces clinical symptoms in mice with CIA which is at least partially mediated by the β -AR. The additive effect of anti-TNF in reducing the clinical scores demonstrates that that mechanism of action of SNS is not primarily mediated by reducing TNF levels. Moreover, anti-TNF potentiating the inhibitory effect of SNS is supporting a combined use of these treatments, or even a combination of SNS with other biologicals, to treat RA, potentially getting more patients closer to remission. In conclusion, the data is providing compelling scientific rationale and pre-clinical evidence that splenic neuromodulation might be a new treatment modality for RA.

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SAT0019

HISTOPATHOLOGICAL CHANGES OF SYNOVIAL TISSUE IN RHEUMATOID ARTHRITIS PATIENTS TREATED WITH TNF INHIBITORS

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Background: Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of synovial tissues [1]. Tumor necrosis factor (TNF)- α is one of the pro-inflammatory cytokines that play a crucial role in the pathogenesis of RA synovitis, and TNF inhibitors (TNFi) were reported to force the RA to go into remission or low disease activity and have brought revolutionary impacts on RA treatment [2]. TNFi have been shown to act on inflammatory cells and form the discoid fibrosis in the sublining layers [3,4]. However, the changes of synovial tissue and the cause of discoid fibrosis in RA patients treated with TNFi has not been determined in detail.

Objectives: The purpose of this study is to demonstrate the histological changes and the types of cells around discoid fibrosis in RA synovium treated with TNFi.

Methods: Synovial tissues were obtained from 30 patients with RA during joint surgeries. 6 patients were treated with TNFi (1 patient with golimumab, 3 patients with etanercept, 2 patients with infliximab). As a control, synovial tissues were obtained from 6 patients who were treated only with csDMARDs (6 patients with MTX). The frozen sections were stained by hematoxylin and eosin (HE). To detect the apoptosis, TdT-mediated dUTP nick end labeling (TUNEL) was performed. The immunohistochemical characterization of the synovial cells was performed by using following antibodies: CD20 and CD3 for detecting B and T lymphocytes respectively, CD163 and CD86 for detecting M1 and M2 macrophage respectively.

Results: In the sections stained with HE, the formation of discoid fibrosis and the other characteristic changes including hydropic degeneration, vacuolation, sclerosis of small vasculature, and the number of multilayered synovial cells was decreased in synovium from RA patients treated with TNFi. In the sections with TUNEL stain, apoptosis of lining cells around the discoid fibrosis was detected in RA synovium treated with TNFi (Figure 1a, 1b). In the sections with immunohistochemistry stain, CD86 expression increased in lining layer of RA synovium treated with TNFi. CD163 positive cells showed diffuse expression in RA synovium treated with TNFi. In contrast, CD20 and CD3 positive cells decreased around discoid fibrosis compared to control sections. These results showed indicated that the types of cells in lining and sublining layers were mainly macrophages and that the apoptosis of macrophages might form the discoid fibrosis in lining layers.